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AN EVALUATION OF THE POTENTIAL USE OF ISONIAZID, ACETYLISONIAZID AND ISONICOTINIC ACID FOR MONITORING THE SELF-ADMINISTRATION OF DRUGS

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1 The possibility of using minute doses of the antituberculosis drug isoniazid (INH) or of its metabolites acetylisoniazid (AcINH) or isonicotinic acid (INA) as innocuous markers for monitoring patient compliance has been investigated.

2 The ingestion of these colourless and tasteless compounds can readily be demonstrated using a sensitive and specific colorimetric method for detecting INA and its metabolite isonicotinylglycine (INAG) in the urine that is rapid and simple to perform.

3 Studies on the kinetics of the urinary elimination of INA and INAG after the ingestion of 6 mg doses of either INH, AcINH or INA by small groups of volunteers indicated the potential suitability of INH or AcINH for monitoring daily or twice-daily self-medication and the appropriateness of INA as a marker for investigating the compliance of drugs prescribed for thrice-daily ingestion.

4 More extensive studies showed that over 99% of the urine samples collected within 18h of dosage with 6 mg INH would give positive results when tested for the presence of INA and INAG, and that doses of 2–6 mg INH could readily be incorporated into capsules or tablets and used as markers for monitoring the ingestion of the antituberculosis or antileprosy drugs dapsone, thiacetazone, ethionamide or prothionamide, or the antihypertensive oxprenolol. Such doses are less than a fiftieth of the normal therapeutic INH dose used in the treatment of tuberculosis.

5 Evidence is presented that INH, AcINH and INA possess most of the characteristics that one would hope to find in a marker for monitoring compliance including very limited inter-individual variability in the rates at which they are converted to the compounds being detected in the urine.

Introduction

Numerous investigations have shown that patients frequently fail to ingest the medicaments prescribed for their treatment (for reviews see Fox, 1962; Porter, 1968; Marston, 1970; Blackwell, 1972; Sackett & Haynes, 1976). Drug ingestion can only be conclusively established by demonstrating the presence of the drug or one of its metabolites in a body fluid such as urine, saliva or blood. However since convenient methods are not available for detecting many drugs, studies of patients' compliance would be considerably facilitated if a suitable marker substance could be identified of widespread applicability for preparing special formulations of 'tagged' tablets or capsules.

There are a number of properties that an ideal marker for monitoring drug compliance should possess. It must be innocuous. It should also be tasteless, colourless and odourless, pharmacologically inert in man, chemically unreactive, cheap and non-bulky. Furthermore the marker, or one of its metabolites, should be capable of being detected in the urine by means of a simple, robust, sensitive and specific procedure. Urine-test methods are probably the most suitable since they are non-invasive and because urinary concentrations of most compounds, particularly if they are relatively polar, are likely to be considerably higher than the concomitant salivary levels. It would therefore be advantageous if the urinary stability of the compound being detected were high and essential that other normal urinary constituents do not interfere with its assay.

The kinetics of the elimination of the compound being detected in the urine, and the dose of the marker administered should be such that the occurrence of both 'false negative' and 'false positive' urine-test results are minimized. The marker should be rapidly absorbed so that positive results are

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obtained soon after ingestion. If the marker is to be incorporated in tablets or capsules prescribed for once-daily self-administration, then a single dose should ensure that urine samples collected up to 24 h give reliably positive results. Soon afterwards, however, and certainly before 48 h, uniformly negative results should be obtained. The ideal half-life for the elimination of a marker for monitoring once-daily self-administration is therefore probably between about 4 and 6 h, since urinary excretion would then decline by between 16- and 64-fold over 24 h. By contrast markers for monitoring the ingestion of drugs that are to be taken several times each day should probably have half-lives of 1–2 h. Furthermore the precision with which urine-test results can be interpreted would be improved if the rate of elimination of the compound being detected in the urine did not vary greatly between individuals and were not appreciably influenced by the co-administration of other substances.

The results of several recent studies (Stark, Ellard, Gammon & Fox, 1975; Ellard & Gammon, 1976; Ellard & Greenfield, 1977) led us to investigate the feasibility of using small doses of the antituberculosis drug isoniazid (isonicotinylhydrazine, INH) or of its metabolites acetylisoniazid (AcINH) or isonicotinic acid (INA) as markers for monitoring drug compliance.

The pharmacology of INH has been extensively investigated in man (Evans, 1968; Peters, 1968; La Du, 1972; Ellard & Gammon, 1976). The most important route for the elimination of INH is by acetylation to AcINH. The rapidity of acetylation of INH is genetically determined in a simple Mendelian fashion, the great majority of individuals being clearly characterized as either 'slow' or 'rapid' acetylators of the drug. INA is formed by hydrolysis of AcINH and then conjugated with glycine to give isonicotinylglycine (INAG). Recent studies (Ellard & Jenner, unpublished observations) have confirmed previous evidence indicating that none of these metabolic routes is inducible.

This report describes studies of the kinetics of the urinary elimination of INA and INAG after the ingestion of 6 mg doses of INH, AcINH and INA by slow and rapid acetylators that were undertaken to provide a quantitative pharmacological basis for their use as potential markers for monitoring the self-administration of drugs. These studies indicated that INH was probably the most suitable marker for monitoring daily self-administration. More extensive studies were then undertaken in which a simple qualitative method for the detection of INA and INAG (Ellard & Greenfield, 1977) was used to assess the positivity of urine samples collected at various times from a larger number of volunteers after the ingestion of 6 mg doses of INH. The potentialities for such a marker were further explored by studies on the kinetics of the excretion of INA and INAG following the ingestion of specially formulated capsules or tablets containing antituberculosis, antileprosy or antihypertensive drugs tagged with 2 or 6 mg doses of INH.

Methods

Dosage with INA, AcINH and INH, and collection of urine samples

INA and INH were purchased from BDH Chemicals, Poole, Dorset, U.K. and recrystallized from water and butan-l-ol, respectively. AcINH was synthesized by the method described by McKennis, Yard & Pahnelas (1956) and recrystallized from butan-l-ol. Capsules containing 6 mg INH plus either dapsone (100 mg), thiacetazone (150 mg), dapsone plus thiacetazone, or prothionamide (125 mg), were prepared by the International Dispensary Association, Amsterdam, the Netherlands. Tablets containing 2 mg INH together with either 40, 80 or 160 mg oxprenolol, or 6 mg INH with 160 mg oxprenolol in a slow-release form were formulated by CIBA Laboratories, Horsham, Sussex, U.K., while May and Baker, Dagenham, Essex, U.K. produced tablets containing 6 mg INH with either 125 mg ethionamide or prothionamide. The INH contents of the capsules and tablets were verified by extracting with methanol and determining INH fluorimetrically (Ellard & Gammon, 1976).

Details of the timing of the complete urine collections made after the ingestion of the marker doses of INA, AcINH or INH by the volunteers who participated in a series of five studies are summarized in Table 1. Informed consent was obtained from each of the 39 volunteers (23 male, 16 female), who were healthy members of the staff of the Medical Research Council Unit for Laboratory Studies of Tuberculosis or the Bacteriology Department of the Royal Postgraduate Medical School with ages and weights ranging from 19–58 years and 45–90 kg, respectively.

In the first study a dose of 6 mg INA and two doses each of 6 mg AcINH and of 6 mg INH were taken dissolved in water by groups of four male volunteers. The taking of each of these doses were separated by an interval of at least a week. The INA dose, and the first of the AcINH and INH doses were taken mid-morning approximately 3 h after a light breakfast and 2 h before lunch. The second AcINH and INH doses were taken late-evening 3–5 h after a substantial meal. The same four volunteers (all slow acetylators) ingested the doses of INA and AcINH, but for the studies with INH the composition of the group was changed to include two slow and two rapid acetylators.

In the second and third studies 6 mg doses of INH dissolved in water were taken by groups of 27–29 volunteers mid-morning (Study II) or in the afternoon some 3 h after a light lunch (Study III). In the fourth study 12 volunteers ingested 6 mg INH mid-morning in a capsule that also contained 100 mg dapsone plus 150 mg thiacetazone. Finally in the fifth study one of the volunteers who participated in the previous four studies (GE) ingested over a period of about 18 months a series of 2 or 6 mg INH doses either dissolved in water; in capsules that also contained dapsone, thiacetazone, dapsone plus thiacetazone or prothionamide, or in tablets containing oxprenolol, ethionamide or prothionamide. All doses were taken mid-morning, with but one exception when a 6 mg INH dose was swallowed immediately after a substantial mid-day meal.

Detection of INA and INAG

Urine samples, after randomization and recoding, were reacted by the qualitative urine-test method described by Ellard & Greenfield (1977). A positive result was indicated by the appearance of a blue, green or grey colour (depending on the natural background colour of the urine sample) within 30 min. Each sample was classified independently by either two or three observers. Where an observer found it impossible to classify a sample as being either definitely 'positive' or 'negative', the result was recorded as 'doubtful'. Initially the observers found it helpful to read the reacted samples against suitable paired blanks which could be prepared by omitting the addition of the cyanide reagent during the reaction sequence. However after a few days they became so adept at reading the tests that this precaution could be dispensed with.

Determination of 'apparent' INA (INA together with INAG)

Duplicate 0.5 ml aliquots of urine were reacted by the method used for the detection of INA and INAG, with the first of each pair serving as a blank and receiving 0.1 ml water instead of 0.1 ml 10% (w/v) aqueous potassium cyanide. Thirty min after reaction the extinctions were determined at 600 nm using a Gilford Microspectrophotometer 300 N equipped with a rapid sampling cuvette and the 'apparent' INA concentration calculated after subtracting the extinction of the blank. The absorption of the chromophore derived from INAG at 600 nm is on a weight for weight basis about 50% of that given by INA with the consequence that since similar amounts of INA and INAG are excreted at all times after dosage with either INH or AcINH, apparent INA concentrations are usually about 1.5 times the true values. Standards (duplicate) consisted of pairs of 0.5 ml aliquots of aqueous 1 μg/ml INA.

Specific determination of INA and INAG

The method used was modified from that described previously (Ellard, Gammon & Wallace, 1972) to increase its sensitivity. A 10 ml sample of urine was pipetted into a large centrifuge tube and extracted with 15 ml butan-l-ol after the addition of 2 ml 4M sodium acetate and 8 g of ammonium sulphate. A 10 ml portion of the extract was then extracted by shaking with 10 ml of ethyl acetate/n-heptane (1:1, v/v) and 2 ml of 4M sodium acetate. Duplicate 0.5 ml

Table 1 Urine samples collected after ingestion of marker doses of INA, AcINH or INH

Study	*Number of volunteers*	*Dose*	*Timing of urine collections (h after dose)*
I	4	6 mg INA	0, 0–1, 1–2, 2–3, 3–4, 4–5, 5–6, 6–7.
	4	6 mg AcINH	0, 0–1, 1–2, 2–3, 3–4, 4–6, 6–8, 8–10. 10–12, 12–21, 21–23, 23–25, 25–27.
	4	6 mg AcINH*	0, 0–10, 10–12, 12–14, 14–16, 16–18, 18–21, 21–23, 23–25.
	4	6 mg INH	0, 0–1, 1–2, 2–3, 3–4, 4–6, 6–8, 8–10, 10–12, 12–21, 21–23, 23–25, 25–27.
	4	6 mg INH*	0, 0–10, 10–12, 12–14, 14–16, 16–18, 18–21, 21–23, 23–25.
II	27	6 mg INH	0, 0–1, 1–2, 2–3, 23.5, 23.5–24.5.
III	29	6 mg INH**	0, 0–1, 17.5, 17.5–18.5.
IV	12	6 mg INH†	0, 0–2, 2–4, 4–6, 23.5–24.5, 47.5–48.5.
V	1	2 or 6 mg INH (series of 23 doses)‡	0, 0–1, 1–2, 2–3, 3–4, 4–6, 6–8, 8–10, 10–12, 12–24.

*Late evening dose; **afternoon dose; all other doses taken mid-morning.
†In capsules; ‡4 in capsules, 11 in tablets; all other doses in aqueous solution.

aliquots of the aqueous sodium acetate extract were then reacted as described previously (Ellard *et al.*, 1972) with the exception that 0.1 ml water was added to one of the pair in place of 0.1 ml 10% aqueous potassium cyanide in order to provide each sample with its own reference 'blank'. Thirty min after reaction the extinction of the extracts were measured at 600 and 620 nm, respectively, and the concentrations of INA and INAG calculated after substracting the appropriate blank values. Standards (prepared in triplicate) consisted of 10 ml aliquots of aqueous solutions containing 1 μg/ml INA and 2 μg/ml INAG, respectively.

Determination of creatinine

Urinary creatinine concentrations were determined by a modification of the alkaline picrate method (Ellard, Gammon, Helmy & Rees, 1974).

Acetylator phenotyping

The acetylator phenotypes of the volunteers who participated in the first and fifth investigation and of 8 of the subjects taking part in the second investigation were established by collecting urine samples 5–6 h after dosage with 40 mg/kg$^{0.7}$ sulphadimidine and determining the ratio of acetylated to free drug (Evans, 1969; Ellard & Gammon, 1977). The acetylator phenotypes of the 27 volunteers who ingested 6 mg isoniazid in the second study were determined by estimating the ratios of AcINH to acid-labile INH in the urine samples collected 2-3 h after dosage. The possibility of phenotyping subjects in this way was suggested by previous studies on the ratio of the excretion of AcINH/acid-labile INH after oral dosage with 10–250 mg INH (Ellard & Gammon, 1976) or intravenous injection of 5 mg/kg of the drug (Ellard, Gammon & Tiitinen, 1973).

The methods used for determining AcINH and acid-labile INH were modified from those described previously (Ellard *et al.*, 1972; Ellard & Gammon, 1976) to increase their sensitivity. A 10 ml sample of urine was pipetted into a large centrifuge-tube, treated with 0.5 ml 2M HCl for 15 min at room temperature to hydrolyze acid-labile INH hydrazones, neutralized with 0.5 ml 2M NaOH and then extracted with butan-l-ol as described above for the determination of INA and INAG. After extracting INA and INAG into 4M sodium acetate, 18 ml of the organic phase was extracted with 6 ml 0.1M HCl. Acid-labile INH was then determined by pipetting duplicate 1 ml aliquots of the 0.1M HCl extract into small stoppered centrifuge tubes and reacting by fluorimetric procedure described previously for INH (Ellard & Gammon, 1976) with the exception that 0.3 ml water was added to one of each pair instead of the salicylaldehyde reagent in order to provide each sample with its own reference blank. The concentrations of acid-labile INH were calculated after substracting the fluorescence of the blank extracts from those of the salicylaldehyde-treated extracts.

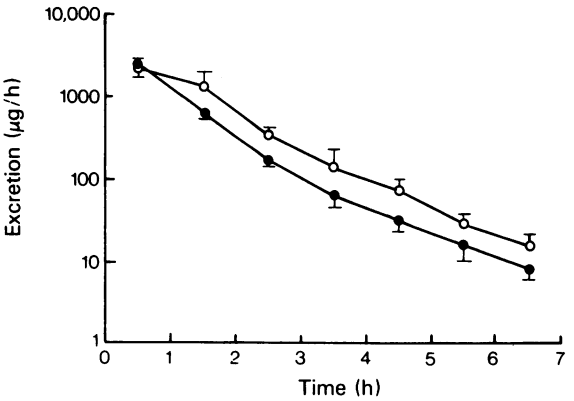

Figure 1 Urinary excretion of INA (●) and INAG (○) after ingestion of 6 mg INA. Points represent geometric means for the four volunteers and bars show ranges of observations.

AcINH was determined by pipetting duplicate 1 ml aliquots of the 0.1M HCl extract into small stoppered centrifuge tubes, heating for 24 h at 60°C to hydrolyze it to INH, and then determining the liberated INH as described above. The concentrations of AcINH were calculated after subtracting the fluorescence of the blank extracts and allowing for the contributions due to INH. Standards (triplicate) consisted of 10 ml aliquots of aqueous solutions containing 0.5 μg/ml INH and 1 μg/ml AcINH, respectively.

Urinary ratios of AcINH/acid-labile INH were bimodally distributed and indicated that the group consisted of 15 slow and 12 rapid acetylators with ratios of 0.83–2.67 (geometric mean 1.37) and 4.65–14.0 (geometric mean 6.74), respectively, and the results obtained for the 8 subjects who were phenotyped by both this and the sulphadimidine method were entirely in accord.

Results

Excretion of INA and INAG after ingestion of 6 mg doses of INA, AcINH and INH (Study 1)

The kinetics of the excretion of INA and INAG after dosage with 6 mg of each of the potential marker compounds are illustrated in Figures 1, 2, 3 and 4 and their cumulative elimination summarized in Table 2.

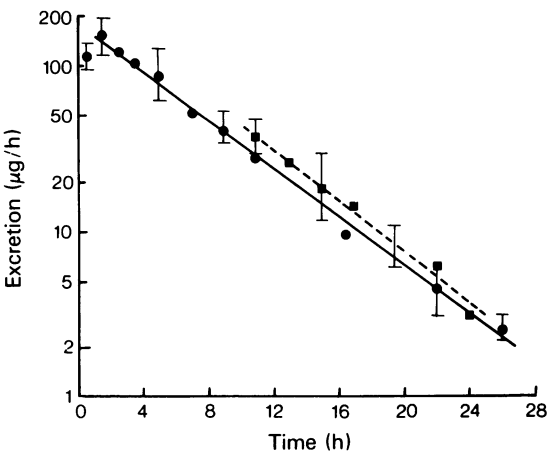

Figure 2 Excretion of INA after ingestion of 6 mg AcINH. Points represent geometric means. First (mid morning) dose (●——●), second (late evening) dose (■---■). For clarity only about half of the ranges of observations for the four volunteers are shown.

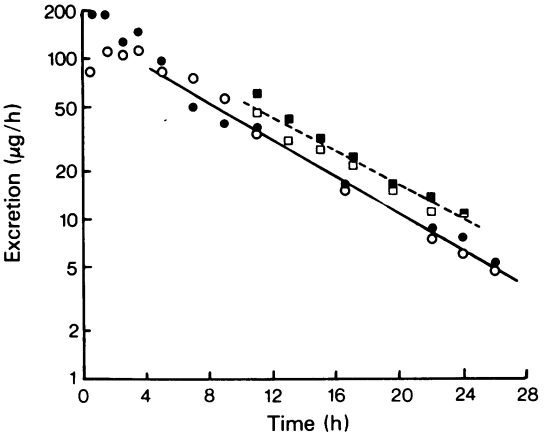

Figure 3 Excretion of INA after ingestion of 6 mg INH. Points represent geometric means for pairs of volunteers. First (mid morning) dose slow (○——○), rapid (●——●) acetylators. Second (late evening) dose slow (□---□), rapid (■---■) acetylators.

INA was extremely rapidly absorbed and conjugated with glycine, maximal urinary excretion of both compounds occurring within 1 h (Figure 1). Over the next 2 h the urinary excretion of INA fell at a rate equivalent to a half-life of about 0.5 h, but thereafter the rate of fall of its excretion declined and by 7 h was equivalent to a half-life of about 1.0 h. The results from each of the four volunteers were remarkably similar. Thus although the mean rates of urinary excretion of INA and INAG declined by about 300- and 150-fold, respectively, during the 7-h period, individual rates of excretion at 6-7 h varied less than 2-fold. The entire administered dose was recovered in the urine as INA and its metabolite INAG (Table 2), the glycine conjugate accounting for 41–47% of the total. The results obtained for volunteer GE were very similar to those obtained some 6 years previously when a 25 mg dose of INA was ingested (Ellard & Gammon, 1976).

AcINH also appeared to be rapidly absorbed since maximal excretion of INA occurred 1–2 h after dosage (Figure 2). Urinary excretion of INA fell exponentially over the period 1–27 h and the results obtained from the four subjects after giving either the first (mid-morning) or second (late-evening) AcINH doses were very similar yielding mean half-lives of 4.1 ± 0.05 and 3.9 ± 0.2 h, respectively. Although the mean rate of INA excretion declined almost 60-fold by 26 h, the individual rates at the end of this period varied by a factor of only about 2-fold. The pattern of urinary excretion of INAG was extremely similar to that of INA and about 40% of the AcINH dose was recovered in the urine as INA plus INAG (Table 2), 37–40% of the INA formed having been conjugated

Table 2 Cumulative excretion of INA plus INAG after oral ingestion of 6 mg INA, AcINH or INH (Study I)

Compound administered	*Dose*	*Individual recoveries*†						*Mean*
Subjects		*GE**	*PJ**	*PD**	*VA**	*BA***	*PA***	
INA	I	97	96	104	108	—	—	101
AcINH	I	35	36	44	48	—	—	41
	II	33	31	42	42	—	—	37
INH	I	33	33	—	—	36	39	35
	II	34	33	—	—	43	45	39

†Percentages of the doses recovered in the urine.
*Slow acetylators, **Rapid acetylators

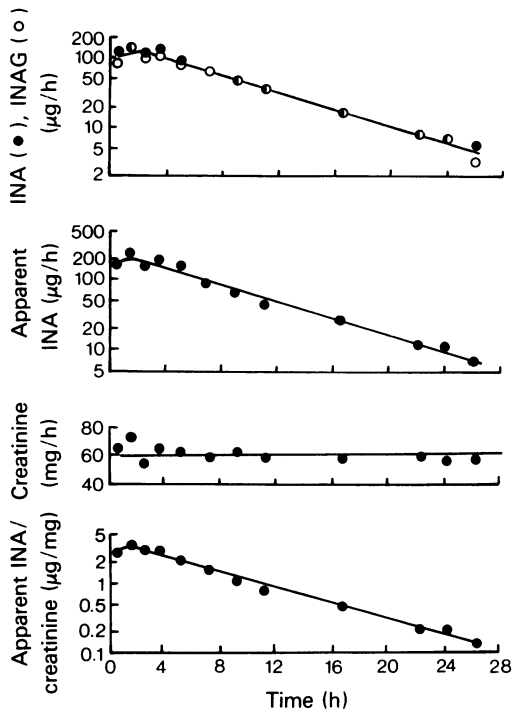

Figure 4 Excretion of INA, INAG, apparent INA, creatinine and ratio of apparent INA/creatinine after ingestion of 6 mg INH. Points represent geometric means for the four volunteers.

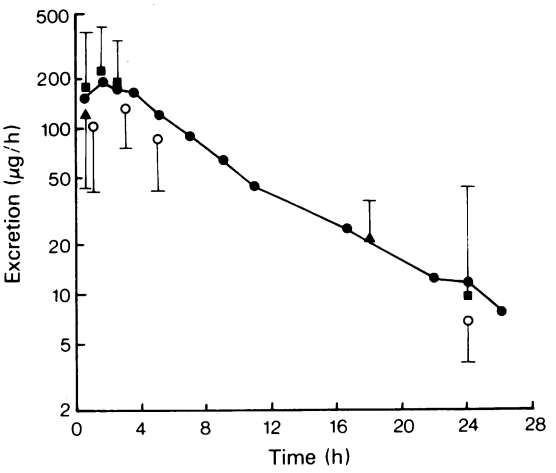

Figure 5 Excretion of apparent INA after ingestion of 6 mg INH. Points represent geometric means and bars show ranges of observations. (●) Study I (4 volunteers), (■) Study II (27 volunteers), (▲) Study III (29 volunteers), (○) Study IV (12 volunteers).

with glycine. The results obtained for the volunteer GE closely resembled those obtained some 6 years previously when doses of 50 and 500 mg AcINH were ingested (Ellard & Gammon, 1976).

It was evident that the absorption of INH was also very swift since maximal urinary excretion of INA and INAG occurred within 2 h (Figures 3 and 4). The two rapid acetylators initially excreted INA more rapidly than the two slow acetylators. This was to have been expected since the major metabolic route for the formation of INA from INH is via AcINH (Peters, Miller & Brown, 1965; Ellard & Gammon, 1976). However from 4 h the excretion of INA by the 2 phenotypes was similar and fell at a rate equivalent to a half-life of 5.2 ± 0.1 h. A broadly comparable pattern of INA elimination (half-life 5.7 ± 0.3 h) was encountered after giving the 4 subjects late-evening doses of INH. Approximately 37% of the INH dose was recovered in the urine as INA plus INAG (Table 2), 39–46% of the INA formed having been conjugated with glycine.

The rates of excretion of INA and INAG after giving INH were virtually identical from 1 h onwards (Figure 4) with the consequence that the estimates of apparent INA were at all times approximately 50% higher than true INA concentrations. Accordingly comparisons of the urinary excretion of INA and its glycine conjugate after dosage with 6 mg INH by the larger numbers of volunteers investigated in Studies III and IV were simplified by measuring apparent INA concentrations instead of specifically determining both INA and INAG. Furthermore,

Table 3 Urinary excretion (mean ± s.e.mean) of INA and INAG (μg/h) after oral ingestion of 6 mg INH by 15 slow and 12 rapid acetylators (Study II)

Time	*Slow acetylators*		*Rapid acetylators*	
(h)	*INA*	*INAG*	*INA*	*INAG*
0–1	125 ± 10	94 ± 11	191 ± 18	145 ± 24
1–2	149 ± 12	147 ± 13	199 ± 19	201 ± 24
2–3	113 ± 7	115 ± 8	162 ± 11	169 ± 12
23.5–24.5	9.5 ± 1.6	9.9 ± 2.0	6.9 ± 1.2	7.4 ± 1.9

because of the constancy in the rates of creatinine excretion, it was possible to obtain an excellent representation of the time pattern of INA and INAG excretion for each subject by calculating the ratio of the concentration of apparent INA to that of creatinine for each urine sample (Figures 4 and 6). Such a procedure was employed in Study V, and would be applicable in circumstances where it might be impossible to guarantee the collection of complete timed urine collections.

Excretion of INA after ingestion of 6 mg doses of INH (Studies II–IV)

The rates of urinary excretion of INA and INAG from 0–1, 1–2, 2–3 and 23.5–24.5 h after dosage with 6 mg INH by the 27 volunteers participating in the second study are summarized in Table 3. During the first 3 h, the rapid acetylators excreted about 1.4 times as much INA and INAG as the slow acetylators, although by 24 h the excretion of both metabolites had fallen to about 75% of that of the slow acetylators. Although these differences were small, they were highly significant ($P < 0.001$). Over the period 2–24.5 h the excretion of INA fell at rates equivalent to a mean half-life of 5.8 h in the slow acetylators (range 4.3–9.4 h) and 4.5 h in the rapid acetylators (range 3.8–5.7 h).

The rates of excretion of apparent INA in studies II–IV after dosage with 6 mg INH are illustrated in Figure 5 together with the more detailed results from the four volunteers in Study I for comparison. The pattern of INA elimination was similar in each of the four studies despite the fact that in Studies I–III the doses were taken dissolved in water while in Study IV they were ingested in a capsule together with dapsone and thiacetazone, and that doses were taken mid-morning in Studies I, II and IV and in the afternoon on Study III. In each study the rate of fall in the excretion of INA over the period from about 3–24 h after dosage was equivalent to a half-life of about 5 h.

Excretion of INA after ingestion of 2 and 6 mg doses of INH in a variety of formulations (Study V)

The urinary excretion of apparent INA after the ingestion of a series of 2 or 6 mg doses of INH either dissolved in water or taken in specially formulated capsules or tablets by subject 1 over a period of about 18 months is illustrated in Figure 6, while the cumulative 24-h excretions of INA plus INAG are summarized in Table 4. The similarity of the results obtained suggests that on every occasion the INH dose was rapidly and completely absorbed and that neither dapsone, thiacetazone, ethionamide, prothionamide or oxprenolol, significantly impaired the absorption of INH or affected the rate at which it was metabolized to INA and INAG. Absorption of

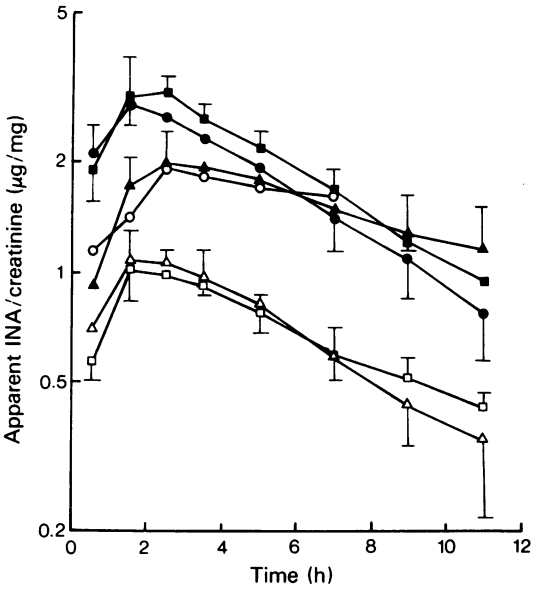

Figure 6 Ratios of excretion of apparent INA to creatinine after ingestion of a series of doses of 2 or 6 mg INH by the volunteer investigated in Study V. Points represent geometric means. For clarity, only the extremes of the ranges of observations are shown. (●) 6 mg in aqueous solution (5 doses), (■) 6 mg in capsule form (4 doses), (▲) 6 mg in tablet form (5 doses), (○) 6 mg in aqueous solution on a full stomach (1 dose), (□) 2 mg in aqueous solution (2 doses), (△) 2 mg in tablet form (6 doses).

INH from the capsules and tablets containing ethionamide, prothionamide or oxprenolol was as rapid as when it was taken in solution. Absorption of INH was however delayed but not impaired when it was taken in tablets containing oxprenolol in a slow-release form or on the one occasion when it was taken in solution on a full stomach.

Table 4 Cumulative excretion of INA plus INAG after oral ingestion of 2 or 6 mg doses of INH (Study V).

INH dose and formulation	*Number of ingestions*	*Recovery**
2 mg solution	2	32 (24, 39)
2 mg tablets	6	39 (32–41)
6 mg solution	6	35 (32–38)
6 mg capsules	4	34 (30–42)
6 mg tablets	5	30 (27–35)

*Percentage dose recovered in the urine, means and ranges of individual results.

Positivity of urine samples collected at various times after ingestion of 6 mg INH

The positivity of the urine samples collected at various time intervals up to 48.5 h from the 39 volunteers investigated in studies II to IV when tested by the qualitative procedure for detecting INA and INAG is shown in Table 5. Extremely few 'false positive' readings (2%) were recorded when the pretreatment samples were reacted, whereas all the urine samples collected from 1–6 h gave uniformly positive results. From about 18 h a significant proportion of the urine samples were classified as negative or doubtful but even 24 h after dosage about 70% of the samples still gave positive readings. By 48 h however the results matched those given by the pretreatment samples. The results showed that in reading the test doubtful samples should be classified as negative. The results obtained in Study V from the urine samples collected from subject 1 up to 12 h after the ingestion of 8 doses of 2 mg INH (in soluble or tablet form) and 15 doses of 6 mg INH (in soluble, capsule or tablet form) confirmed these conclusions, all pretreatment samples being classified as negative and all other samples as positive.

Samples could also be classified according to their apparent INA concentrations (Table 5), the most efficient criterion being to define positive samples as those with apparent INA concentrations of greater than 0.1 μg/ml. However such a procedure was no better at discriminating between post- and pre-treatment samples than reading by eye. Creatinine concentrations ranged from 0.11 to 3.62 mg/ml (geometric mean, 0.77 mg/ml) and at each time point there was a highly significant correlation ($P < 0.001$) between INA and creatinine concentrations, so that variations in INA/creatinine ratios were much more limited than the spread in INA concentrations (Table 5). Thus estimating INA/creatinine ratios was largely able to compensate for the effects of diuresis in a way that had been utilized previously to monitor the ingestion of dapsone by measuring urinary dapsone/creatinine ratios (Ellard *et al.*, 1974), and the most efficient method of discriminating between post- and pre-treatment samples was to use a ratio of greater than 0.1 μg apparent INA/mg creatinine as a criterion of positivity (Table 5).

Sensitivity of methods for the determination of INA, INAG, INH and AcINH

The sensitivity of the analytical methods employed to determine the urinary concentrations of these four compounds is primarily determined by the magnitude of the normal blank values (Ellard *et al.*, 1972). Analysis of the results obtained in this investigation on pretreatment urine samples showed that the urinary blank values were reduced between 3- and 5-

Table 5 Positivity of urine samples collected at various times after ingestion of 6 mg INH

Period after dose (h)	*Number of samples*	*% Positive by eye*	*Apparent INA** (μg/ml)	*% > 0.1 μg/ml*	*Apparent INA/creatinine** (μg/mg)	*% > 0.1 μg/mg*
0	68	2 (11)**	0† (0–0.13)	1	0† (0–0.10)	0
0–1	56	100	1.39 (0.32–9.18)	100	2.77 (0.78–9.80)	100
0–2	12	100	0.93 (0.21–4.63)	100	1.37 (0.61–4.06)	100
1–2	26	100	2.13 (0.58–10.58)	100	4.26 (2.41–10.95)	100
2–3	25	100	2.12 (0.55–6.76)	100	3.57 (2.08–7.77)	100
2–4	12	100	1.78 (0.35–6.40)	100	2.05 (0.96–4.56)	100
4–6	12	100	1.35 (0.26–5.65)	100	1.46 (0.68–3.88)	100
17.5	28	97 (1)	0.46 (0.05–1.65)	93	0.48 (0.15–0.82)	100
17.5–18.5	29	88 (6)	0.24 (0.01–1.79)	86	0.38 (0.09–0.81)	97
23.5	26	84 (11)	0.17 (0.05–0.66)	73	0.18 (0.08–0.51)	92
23.5–24.5	35	71 (10)	0.14 (0.03–07.4)	71	0.15 (0.05–0.53)	80
47.5–48.5	12	0 (7)	0† (0–0.06)	0	0† (0–0.04)	0

*Geometric means and ranges **Percentages of doubtful readings †Median values

fold by correcting the colour or fluorescence of reacted samples for that given by paired blanks in which an essential reagent (potassium cyanide or salicylaldehyde) had been omitted, and that as a consequence the methods were capable of reliably measuring concentrations of down to about 0.04 μg/ml INA, 0.06 μg/ml INAG, 0.03 μg/ml INH and 0.2 μg/ml AcINH.

Discussion

The only convincingly innocuous marker that appears to have been seriously investigated in the past is the vitamin riboflavin, which can be detected and quantitated in urine from its fluorescence when exposed to ultraviolet light (Hobby & Deuschle, 1959; Deuschle, Jordahl & Hobby, 1960). There are however a number of major drawbacks to its use as a marker for monitoring compliance. Thus riboflavin is present in many foodstuffs, is a normal urinary constituent and is present in many proprietary vitamin preparations. Moreover, in order to obtain urinary concentrations of riboflavin in excess of normal levels for 12–15 h, relatively large amounts of riboflavin need to be taken (0.8 mg/kg). Riboflavin is also highly coloured and, especially during the first few hours after its ingestion, imparts a strong and noticeably unnatural yellow hue to the urine. In addition the fluorimetric assay technique described for its quantitation (Hobby & Deuschle, 1959) is complicated and time consuming, and suffers from interference from porphyrin, a degradation product of haemoglobin sometimes present in the urine.

By contrast, the characteristics summarized in Table 6 indicate that small doses of either INH, AcINH or INA possess most of the attributes that one would hope to find shown by a marker for monitoring compliance. There is extremely convincing evidence to indicate that such doses of INH will be innocuous. World-wide experience during the past two decades in the treatment of tens of millions of tuberculosis patients with daily doses of approximately 5 mg/kg INH, given together with various other antituberculosis drugs (see Fox, 1972; Girling, 1978) testifies to isoniazid's safety and excellent tolerance when given for periods of a year or more at a daily dose approximately 50 times greater than that envisaged for its use as a marker. Furthermore, large studies carried out in Britain and the U.S.A., with follow-up periods of 10–20 years, have provided no evidence of a carcinogenic effect of such isoniazid treatment in man (Stott, Peto, Stephens, Fox, Sutherland, Foster-Carter, Teare & Fenning, 1976; Glassroth, White & Snider, 1977).

Studies on the urinary elimination of INH and its metabolites indicate that in slow acetylators 50–60% of the INH dose is converted to AcINH and about 30% to INA, whereas in rapid acetylators the proportions are 80–90% and 40–50%, respectively (Peters Miller & Brown, 1965; Ellard & Gammon, 1976). The excellent tolerance of therapeutic doses of

Table 6 Suitability of small doses of INH, AcINH or INA for monitoring compliance

Characteristics of an ideal marker	*INH*	*AcINH*	*INA*
Innocuous	+	+	+
Tasteless	+	+	+
Colourless	+	+	+
Odourless	+	+	+
Readily available	+	−	+
Chemically inert	−	+	+
Pharmacologically inert in man	+	+	+
Readily detectable*	+	+	+
Small pharmacokinetic variability†	+	+	+
Suitable for monitoring:			
Thrice daily treatment (positive 6 h)	1 mg	1 mg	7 mg‡
Twice daily treatment (positive 9 h)	2 mg‡	2 mg	−
Once daily treatment (positive 12 h)	3 mg‡	3 mg	−
Once daily treatment (positive 18 h)	6 mg‡	9 mg	−
Once daily treatment (positive 24 h)	13 mg	25 ‡	−

*As the metabolites INA and INAG in the urine
†Between different subjects in the elimination of INA and INAG
‡Most suitable marker

INH therefore strongly indicates that marker doses of its metabolites AcINH and INA are also likely to be innocuous in man. Such a conclusion is reinforced by evidence from experimental studies in the mouse, dog and monkey that the toxicity of INH is probably associated with the presence of its partially acylated hydrazine group (Bernstein, Lott, Steinberg & Yale, 1952; Schmidt & Hoffman, 1953; Yard & McKennis, 1962). Thus in each of these species AcINH was much better tolerated than INH. Single doses of 250–500 mg AcINH or INA have also been ingested by volunteers during the course of various studies on their pharmacology without any apparent side-effects (Yard & McKennis, 1962; Peters *et al.*, 1965; Mitchell, Thorgeirsson, Black, Timbrell, Snodgrass, Potter, Jollow & Keiser, 1975; Boxenbaum & Riegelman, 1976; Ellard & Gammon, 1976).

INH, AcINH and INA are tasteless, colourless and odourless so that special formulations containing small amounts of either compound should be indistinguishable from standard preparations. Moreover, at the dose sizes contemplated (about 6 mg), their addition would increase the bulk of most tablets to only a minor extent. Another consideration favouring the use of such compounds as markers is that both INH and INA are relatively inexpensive materials that are widely available in a pure form, and although AcINH cannot at present be purchased commercially it can readily be synthesized by acetylation of INH using the methods originally described by Fox & Gibas (1953) or Yale, Losee, Martins, Holsing, Perry & Bernstein (1953) or their modifications (McKennis *et al.*, 1956; Olson, Dayton, Israili & Pruitt, 1977).

Although AcINH and INA are chemically inert, INH has the propensity of reacting with aldehydes and ketones. Providing sugars such as lactose are avoided (Bailey & Abdou, 1977), it should be possible to prepare suitable isoniazid-tagged formulations of most drugs; indeed commercial formulations of isoniazid combined with drugs such as thiacetazone, ethionamide, prothionamide and rifampicin have been widely used in the treatment of tuberculosis and have displayed the same excellent keeping properties characteristic of the separately formulated drugs. In situations where sugar coated tablets were required, the INH marker might be incorporated into either an outer film or 'spot'. At normal doses (5 mg/kg) INH, and presumably also its metabolites AcINH and INA, appear to be devoid of pharmacological activity in man.

The colorimetric method described for the detection of the INH metabolites INA and INAG is simple, robust, specific, sensitive and rapid. The procedure is a convenient modification (Ellard *et al.*, 1972; Ellard & Greenfield, 1977) of the method originally used by Nielsch (1958) for estimating INA by the König reaction, that requires only 0.5 ml urine, and simply involves noting whether or not a blue colour develops after the sucessive addition of pH 5 acetate buffer, cyanide, chloramine-T and barbituric acid. Both INA and INAG are stable in urine and samples can be stored without refrigeration prior to testing, although the addition of a crystal of thymol to prevent potential bacterial contamination is recommended.

Quantitative studies demonstrated that neither the timing recommended for the addition of the reagents (at 15 s intervals) nor that suggested for reading the test (30 min) was critical. Thus colour formation appeared to be independent of ambient temperature over the range 4–37°C, was 90–95% complete within 15 min, maximal by 30 min, and no significant fading occurred over the next 30 min. Since cyanogen chloride is generated during the reaction sequence, the procedure should if possible be carried out in a fume cabinet, although a convenient alternative utilised in these studies was to react racks containing 16 samples every 4 min (4 samples each minute) and then to place them in a cupboard for colour development. Although Nielsch (1958) originally recommended that reacted samples should be placed in the dark, in our hands light did not appear to affect colour formation.

INA and INAG are not normal urinary constituents and since the method used for their detection is highly specific, it is very likely that positive results will only be given by urine samples from patients ingesting INH. The sensitivity of the urine-test procedure was revealed by the finding that 96% of the samples collected in this investigation with INA and INAG concentrations of between 0.3 and 0.5 μg/ml were classified by eye as positive, as were over 99% of those with concentrations of 0.5–1.0 μg/ml and all those with concentrations of greater than 1 μg/ml. Another favourable trait of the qualitative procedure is the speed with which samples can be tested. Thus a single person can conveniently react batches of 120 samples over a 30 min period using automatic pipettes for the addition of the reagents, and read the results over the following 30 min. When tested in this way the total processing time per sample is only about 1 min. If greater sensitivity or precision is required the ratio of the concentrations of apparent INA/creatinine can be determined at a throughput equivalent to about 4 min per sample.

An additional feature of this investigation which encourages the use of INH as a marker for monitoring patient compliance has been the very limited inter-individual differences in the excretion of INA and INAG encountered after giving 6 mg doses of either INA (Figure 1), AcINH (Figure 2 and Table 2) or INH (Figures 3 and 5, Tables 2 and 3). The reason why the acetylator phenotype of the volunteers played such a minor role in influencing the excretion of INA and INAG after the ingestion of

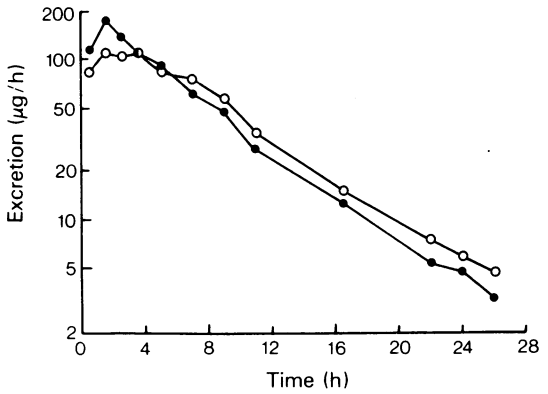

Figure 7 Excretion of INA by volunteers GE and PJ (slow acetylators) after ingestion of 6 mg doses of INH (○) and AcINH (●). Points represent geometric means. The results for AcINH have been multiplied by a factor of 1.31 to allow for its higher molecular weight.

INH (Table 3) can readily be understood from the similarity in both the time course (Figure 7) and cumulative excretion (Table 2) of the two metabolites by the slow acetylators investigated in Study I after the ingestion of either INH or AcINH, the latter situation being equivalent to giving INH to an infinitely rapid acetylator of the drug. The small differences between slow and rapid acetylators in the pattern of INA and INAG excretion (Table 3) probably explains the restricted inter-individual variability in the excretion of apparent INA from 1-18 h after the ingestion of 6 mg doses of INH by the larger numbers (12–29) of volunteers investigated in Studies II–IV (Figure 5). Thus individual results were always less than 2-fold above or below the group means. An analysis of the urine-test results of samples collected from 17.5–24.5 h, when their positivity was beginning to falter (Table 5), failed to reveal a significant relationship between negativity and either urine flow rate or acetylator phenotype. Finally, the stable pattern of excretion of INA and INAG displayed by the volunteer investigated in Study V after swallowing a series of 23 INH doses taken over an 18 month period, either in solution, capsules or tablets (Figure 6 and Table 4), indicates that the incorporation of marker doses of either INA, AcINH and INH into special formulations are likely to provide extremely reliable methods for monitoring individual drug ingestion.

Estimates of the sizes of suitable marker doses of INH, AcINH and INA for monitoring thrice-, twice-, and once-daily treatment are shown in Table 6. These estimates are based on the kinetics of excretion of INA by the four volunteers investigated in Study I after the ingestion of mid-morning doses of 6 mg INH (Figure 3), AcINH (Figure 2) and INA (Figure 1), and the percentages of positive readings given by the qualitative urine-test procedure on urine samples collected in Studies II–IV after dosage with 6 mg INH (Table 5). Examination of the latter data suggested that over 99% of the urine samples collected during the period 0–18 h after dosage with 6 mg INH would have given positive urine-test readings, while the results illustrated in figure 3 indicate that 18 h after such a dose the urinary excretion of INA by the 4 volunteers investigated in Study I averaged about 13.5 μg/h. From the kinetics of the urinary excretion of INA after dosage with 6 mg of each of the potential marker substances, it was calculated that such levels of urinary excretion could be maintained for periods of 6, 9, 12, 18 and 24 h, respectively, by the doses of INH, AcINH or INA, shown in Table 6. In making such calculations it was assumed that over the dose ranges considered the kinetics of the formation of elimination of INA and INAG were not dose-dependent. The validity of such an assumption is apparent from the similarity in the pattern of the excretion of these metabolites after dosage with 2 or 6 mg INH (Figure 6 and Table 4) and a comparison of the results obtained in Study I when volunteer GE ingested 6 mg doses of AcINH and INA with those from a previous investigation when doses of 250–500 mg had been ingested (Ellard & Gammon, 1976).

INA should be the most suitable marker for monitoring thrice-daily self-medication and it was calculated that a dose of 7 mg INA ought to give reliably positive urine test results for a period of 6 h. Doses of 1 mg INH or AcINH could also be used for this purpose but would be less satisfactory since the slower decline in INA elimination (Figures 2 and 3) would be likely to result in a significant proportion of urine samples collected beyond 12 h giving positive results. By contrast doses of either INH or AcINH would be much more suitable for monitoring twice- or once-daily treatment. In the great majority of situations INH would be the marker substance of choice in view of its ready availability and the wealth of previous experience of its use in the treatment of tuberculosis. A dose of 2 mg INH should be eminently suitable for monitoring twice-daily treatment since all samples collected within 9 h of dosage should give positive urine-test results, while the great majority of samples collected beyond 24 h are likely to be negative.

The investigations described in this paper have demonstrated conclusively that 6 mg doses of INH provide an extremely satisfactory marker for monitoring daily self-administration and that such doses can be readily incorporated into capsules or tablets containing antituberculosis or antileprosy drugs such as dapsone, thiacetazone, ethionamide and prothionamide, or the antihypertensive oxprenolol (Figure 6). The results summarized in

Table 5 indicate that in the period up to 18 h after the ingestion of such a dose over 99% of the urine samples should give positive readings, while by 48 h all samples will be negative.

The possibility was considered that the repeated ingestion of marker doses of INH by an individual with unsuspected tuberculosis might lead to the selection of INH-resistant tubercle bacilli and thereby prejudice subsequent treatment with INH-containing regimens. The risk of this happening can however be greatly reduced by giving marked formulations for only relatively limited periods of time and not employing individual INH doses in excess of 0.15 mg/kg. Such a dose, which is unlikely to be surpassed in an adult ingesting 6 mg INH, resulted in plasma INH concentrations in a slow acetylator that exceeded the minimal inhibitory concentration against *M. tuberculosis* (about 0.1 μg/ml) for less than 2h (G.A. Ellard, unpublished results). In many situations a marker dose of 3 mg INH, giving reliably positive results for 12h, would be entirely satisfactory since one could recommend that the daily dose of the medicament be taken first thing each morning (say 07.00h) and then monitor compliance by means of a surprise home visit to collect a urine sample at any time before 19.00 h in the evening. The potential antituberculosis activity of marker doses of INH can however be minimized by giving only occasional doses of the tagged formulation by means of suitable calendar packs and then testing urine samples collected in the period immediately after such doses should have been ingested (Stark *et al.*, 1975). Finally, if one wanted a marker that was devoid of antituberculosis activity and would give reliably positive results for a full 24 h, one could employ a 25 mg dose of AcINH.

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